Serial Number: 09/534,509

Group Art Unit: 1633

MARKED-UP VERSION OF AMENDMENTS

IN THE SPECIFICATION:

Table 2 on page 30 has been replaced by the following Table:

	RNA-D	Aspirin	DNA-CT	DNA-EC	t-RNA
M	~~59,33~~ ~~59,73~~	~~38.66~~ ~~38,66~~	~~54,45~~ ~~54,45~~	~~36,93~~ ~~36,93~~	~~52,23~~ ~~52,23~~
+-m	~~4,24~~ ~~4,24~~	~~6,71~~ ~~6,71~~	~~3,76~~ ~~3,76~~	~~1,88~~ ~~1,88~~	~~8,13~~ ~~8,13~~
		P<0.02	P<0.2	P<0.01	P>0.5

Table 3 on page 31 has been replaced by the following Table:

	RNA 0,1%	RNA 1x10^{-2} %	RNA 1x10^{-3}%	RNA 1x10^{-4} %	RNA 1x10^{-5}%
M	~~78,58~~	~~53,08~~	~~28,88~~	~~43,35~~	~~14,23~~ ~~14,23~~
+-m	~~7,51~~ ~~7,51~~	~~3,23~~ ~~3,23~~	~~1,63~~ ~~1,63~~	~~10,3~~ ~~10,3~~	~~4,98~~ ~~4,98~~
		P<0.01	P<0.001	P<0.01	P>0.001

Table 4 on page 31 has been replaced by the following Table:

Conc. 0.1%	RNA-P	RNA-PN	RNA-F
M	~~84,09~~ ~~84,09~~	~~45,96~~ ~~45,96~~	~~57,9~~ ~~57,9~~
+-m	~~3,77~~ ~~3,77~~	~~8,96~~ ~~8,96~~	~~9,58~~ ~~9,58~~
		P<0.001	P<0.02
Conc. 1x10^{-2} %	RNA-P	RNA-PN	RNA-F
M	~~71,91~~ ~~71,91~~	55.44	~~60,90~~ ~~60,90~~
+-m	~~8,45~~ ~~8,45~~	~~8,04~~ ~~8,04~~	~~10,39~~ ~~10,39~~
		P<0.2	P>0.5
Conc. 1x10^{-3} %	RNA-P	RNA-PN	RNA-F
M	~~29,76~~ ~~29,76~~	~~3,72~~ ~~3,72~~	~~18,26~~ ~~18,26~~
+-m	~~5,36~~ ~~5,36~~	~~2,4~~ ~~2,4~~	~~5,46~~ ~~5,46~~
		P<0.001	P<0.1

Serial Number: 09/534,509 Group Art Unit: 1633

REMARKS

By the present supplemental amendment, Tables 2-4 on pages 30-31 of the specification have been amended to replace commas by periods in scientific notation of numbers, as shown in the marked-up version above. These changes had erroneously been omitted in the amendment filed on September 13, 2001.

Also, a marked-up copy of pages 27-28, 30-33, 47, 49, and 53-55 of the original specification showing the changes made by the amendment filed on September 13, 2001 is attached to this supplemental amendment.

Applicant's representative regrets and apologizes for the errors and the lack of marked-up copy of changes to the specification in the amendment filed on September 13, 2001. It is submitted that the handwritten marked-up versions are easier read than word processing marked-up version because most changes relate to commas and periods.

In the event there is, in the Examiner's opinion, any outstanding issue and such issue may be resolved by means of a telephone interview, the Examiner is respectfully requested to contact the undersigned attorney at the telephone number listed below.

Serial Number: 09/534,509 Group Art Unit: 1633

In the event this paper is not considered to be timely filed, the Applicants hereby petition for an appropriate extension of the response period. Please charge the fee for such extension and any other fees which may be required to our Deposit Account No. 01-2340.

Respectfully submitted,

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VERSION OF AMENDMENTS F. 9/13/01

Table 1.

Chemical Analysis of Yeast RNA Preparations

Type	RNA-P	RNA-D	RNA-F	RNA-PN
Nitrogen content %	15.49	15.16	14.16	14.65
Phosphorus content %	9.05	8.6	8.2	8.54
Biuret reaction	(-)	(-)	(+)	(-)
DNA content %	1	1.1	1.2	1.1

The tested RNA (RNA-P and RNA-D) had the following properties as shown in Table 1: N ≥ 14.7%, P(total) ≥ 8.6%, protein (biuret reaction) – negative, DNA (colometric) - 2.0%, sugars (chromatography) – negative, polysaccharides (biological test) – negative.

Example 1.2. Absence of Toxicity

We established that yeast RNA-P and RNA-D are non-toxic. Single or multiple doses of yeast RNA in bio-active amounts (250 to 500 mg per 1 kg of body weight), taken intra-abdominally, did not lead to substantial changes in the quantity of peripheral lymphocytes in mice. Such changes would be a characterizing indicator for endotoxines.

Analogous results were obtained for intravenous introduction of nucleic acids. We tested variations in the quantity of peripheral leukocytes in rabbits 1-3 hours after 100 mg yeast RNA-P or RNA-D solution was injected intravenously. Intravenously injected solution of 0.85% NaCl was used as the standard of non-toxicity. It was demonstrated that, analogously to the standard, an injection of yeast RNA-P or RNA-D does not cause a variation in the number of leukocytes within 3 hours of the introduction. In animals, which took ~~0,85%~~ 0.85 solution of NaCl, the quantity of leukocytes was equal to 13000±980, while those, who had RNA-P or RNA-D, showed accordingly 12700±850 and 12900±980, which is not abnormal. When the rabbits received injections of 10 mg of proteus polysaccharide, the quantity of leukocytes decreased in 1 hour from 13050±1100 to 2900±210, and remained at that level while the test lasted (3 hours). These results prove

the non-toxicity of yeast RNA. Further, when 100 mg of yeast RNA-P or RNA-D per 1 kg of body weight was given to rabbits intravenously, no acute-phase C-reactive protein was determined, which indicates that there was no endotoxic action.

In addition, yeast RNA is not pyrogenic, which was shown on rabbits. Temperatures were taken 4 times a day, with 2-hour intervals, in a group of rabbits for 2 days. On the third day, the rabbits were injected with 0.85% of NaCl, and the temperatures were taken again 1, 2, and 3 hours after the injection. On the sixth day, the rats were divided into 3 groups, two of which received intravenously 100 mg of RNA-P and RNA-D, respectively. The temperatures were taken again. The control animals showed temperature fluctuations within 0.1° to 0.4°C. The tested animals had temperatures fluctuating within the same limits: 0.1° to 0.4°C. These results prove the non-pyrogenicity of yeast RNA.

Example 2. Anti-Inflammatory Action of Nucleic Acids Based on the Model of Thrombocyte Aggregation *in Vitro*

We studied the anti-inflammatory action of nucleic acids on the model of thrombocyte aggregation in vitro by the method of Born (**Born L.V.R. The aggregation of blood platelets by diphosphate and its reversal, Nature, V.94, P.327, 1962**). Venous human blood was taken in silicon tubes of *Becton Dickson*, which contained a 3.8% solution of sodium citrate. In order to receive thrombocytic-rich plasma, citrate blood was centrifuged at 1500 rev/min for 7 minutes. Plasma free of thrombocytes was obtained by centrifuging ~~2,0~~ 1.0 ml of plasma taken from medium layers for 15 minutes at 3000 rev/min. We counted the number of thrombocytes in the thrombocytic-containing plasma, which was later diluted by the thrombocyte-free plasma to the final concentration ~~200,0-300,0~~ 200.0 300,0 $x10^8$/l.

An Aggregometer produced by "Tromlite" (Poland) was used for thrombocyte aggregation. In order to induce aggregation, arachidonic acid was diluted in Michaelis

per tube, which contained thrombocytic plasma, was also tested as a standard anti-inflammatory agent.

The test results are shown on Table 2 below.

Table 2.

Influence of Nucleic Acids and Aspirin on the Aggregation of Thrombocytes Induced by Arachidonic Acid

	RNA-D	Aspirin	DNA-CT	DNA-EC	t-RNA
M	59,73	38,66	54,45	36,93	52,23
+-m	4,24	6,71	3,76	1,88	8,13
		P<0.02	P<0.2	P<0.01	P>0.5

The test results showed that nucleic acids in the concentration $1x10^{-2}$ % inhibit aggregation of thrombocytes induced by arachidonic acid. Further, Yeast RNA-D in the concentration $1x10^{-2}$ % inhibited aggregation of the induced thrombocytes almost twice as effectively as aspirin (38.66%): yeast RNA-D showed 59.73% and transport *E.coli* RNA had 52.23%. DNA from chicken erythrocytes acted at the same level as aspirin (~~36,93~~ 36.93%), while DNA from cattle thymus inhibited aggregation of thrombocytes by 54.45%, which is almost at the level of yeast RNA. Since DNA always contain a significant amount of RNA, it is probable that the inhibiting effect of DNA can be attributed to the RNA contained in DNA.

Further, an analysis of the influence of different concentrations of yeast RNA on the aggregation of induced thrombocytes showed that yeast RNA was effective in a wide range of concentrations from 0.1% to $1x10^{-5}$ % and inhibited aggregation by ~~78,5~~ 78.5% and ~~14,2~~ 14.2%, as shown in Table 3 below.

Table 3.

Concentration-Dependence of the Influence of Yeast RNA-D on the Aggregation of Thrombocytes Induced by Arachidonic Acid

	RNA 0,1%	RNA $1x10^{-2}$ %	RNA $1x10^{-3}$ %	RNA $1x10^{-4}$ %	RNA $1x10^{-5}$ %
M	78,58	53,08	28,88	43,35	14,23
+-m	7,51	3,23	1,63	10,30	4,98
		P<0.01	P<0.001	P<0.01	P>0.001

Still further, it was demonstrated that the inhibiting effect on aggregation depends on the purity of yeast RNA and its sodium salt, as shown on Table 4 below.

Table 4.

Influence of Yeast RNA-P, -PN and -F on the Aggregation of Thrombocytes Induced by Arachidonic Acid

Conc. 0.1%	RNA-P	RNA-PN	RNA-F
M	84,09	45,96	57,90
+-m	3,77	8,96	9,58
		P<0.001	P<0.02
Conc. $1x10^{-2}$ %	RNA-P	RNA-PN	RNA-F
M	71.91	55.44	60.90
+-m	8,45	8,04	10,39
		P<0.2	P>0.5
Conc. $1x10^{-3}$ %	RNA-P	RNA-PN	RNA-F
M	29,76	3,72	18,26
+-m	5,36	2,40	5,46
		P<0.001	P<0.1

Table 4 shows that RNA-F containing protein admixtures and lower levels of nitrogen and phosphorus content acted less effectively in the range of concentrations from $1x10^{-1}$ % to $1x10^{-3}$ %. For example, at its highest concentration, RNA-F inhibited thrombocytic aggregation by 57%, whereas at its lowest concentration, inhibition was only 22.7%. At the same time, well-purified RNA-P inhibited thrombocytic aggregation by a third more effectively, accordingly, by 84% and 29.7%. Also, when RNA was transformed into its sodium salt, the anti-aggregate properties decreased dramatically. Thus, RNA-PN, at its highest concentrations, was only half as effective (44.4%) as the

acid form, while at its lowest concentration, RNA-PN did not show any anti-aggregate properties.

Therefore, based on the model of aggregation of thrombocytes induced by arachidonic acid, it was demonstrated that RNA compounds and, especially, purified yeast RNA, have pronounced anti-aggregate properties in a wide range of concentrations, which indicates their anti-inflammatory action.

Example 3. Anti-Inflammatory Action of Nucleic Acid Based on the Model of Erythrocyte Membrane Stabilization *in Vitro*

The membrane-stabilizing and anti-radical actions of nucleic acids were evaluated in rat erythrocytes in tests *in vitro*. Erythrocytal membranes were damaged by nitrite anion, a stable metabolite of nitric oxide, which causes oxide injuries in the protein (especially, hemoglobin) and lipid components of the membrane.

In order to evaluate the membrane-stabilizing action of nucleic acids against the influence of free radicals, we calculated the acid resistance of normal rat erythrocytes separated from blood plasma. Rat erythrocytes were rinsed thrice in the cold (4°C) solution of ~~0,15~~ 0.15M of NaCl. The layers of leukocytes and thrombocytes were removed. Acid lysis of the remaining erythrocytes was induced by adding 10 µl of the suspension, which was diluted to the concentration of erythrocytes (~~0,7~~ 0.7x10^6 cells per 1 ml of isosmotic medium), and which contained ~~0,14~~ 0.14M of NaCl, 0.01M of the citrate-phosphate buffer pH=2.5, different concentrations (10 or 100 µg) of nucleic acids, and a stable concentration of nitric sodium, 250 µg per 1 ml, to initiate the oxide damage of erythrocytes.

Erythrocytal lysis was initiated by adding 1 ml ~~0,004~~ 0.004N HCl; changes in existence were recorded at 750 nmol. The method of calculation is explained in Example 6.3. It was demonstrated that yeast RNA-D in the doses of 10 and 100 µg increased the level of total resistance of the erythrocytes from 288 units (control value recorded for the influence

of $NaNO_2$ without yeast RNA) to 449 units (yeast RNA concentration 10 μg) and 437 units (yeast RNA concentration 100 μg), which is close to norm (475 units). RNA-PN increased total resistance to 328 units in the dose of 10 μg and to 415 units in the dose of 100 μg. RNA-P increased total resistance to 315 units in the dose of 10 μg and to 462 units in the dose of 100 μg (maximally close to the normal level of this indicator). RNA-F increased total resistance to 338 units in the dose of 10 μg and, on the contrary, somewhat decreased (to 271 units) in the dose of 100 μg.

DNA-CT increased total resistance to 338 units in the dose of 10 μg and to 654 units in the dose of 50 μg (which is double the control value and even greater than norm (without harmful influence of $NaNO_2$)). In the dose of 100 μg, however, its effect was the opposite – membrane-stabilizing, which was shown by a decreased total resistance to 158 units, which is almost half the control value.

DNA-EC in the dose of 100 μg did not change acid resistance of erythrocytes in our oxide-damage model. In the dose of 10 μg, it increased acid resistance to 408 units, which is a little lower than the calculated protector action of RNA-D (449 units in the dose of 10 μg).

Therefore, exogenous DNA, regardless of their origin, have significant anti-stabilizing influence on cellular membranes. Since they damage cellular membranes, they cannot be used as drugs or food supplements.

The preparation of t-RNA in both doses (10 μg to 279 units and 100 μg to 296 units μg) did not influence the acid resistance of erythrocytes.

The tests show that yeast RNA, when tested in vitro, shows membrane-stabilizing and anti-radical properties which depend on its form, origin, and purity. Well-purified yeast RNA-P, whose anti-inflammatory properties were studied more in detail, showed the best effectiveness.

Example 5.3. Action of Yeast RNA on the Activity of NOS in Ischemia Cases

The tests were conducted on rats with an infarct of myocardium experimentally induced by occlusion of the coronary artery for 30 minutes. Blood was taken from the coronary artery and from the heart, which was divided into the intact zone, border zone, and infarction zone. The activity of NOS ferment was measured in different heart zones and in blood. Also, we measured the contents of free arachidonic acid (heart and blood) and products of its oxidizing metabolism (blood). The test results are shown in Table 11 below.

Table11.

Action of Yeast RNA on the Activity of NOS in Rat Heart in Ischemia (in picomol per 1 mg of protein; M+-m; n=5)

	Ischemia 30 min (Control)				Ischemia 30 min+Yeast RNA		
	Intact (norm)	Border zone	Infarction zone	Together	Border zone	Infarction zone	Together
M +-m	46.500 7.000	259.315 0 60.683	185.626 48.635	129.655 30.341	59.634 11.649	115.122 40.509	122.635 0 26.413
P1		<0.01	<0.05	<0.2	<0.5	<0.1	>0.05
P2					<0.2	<0.5	<0.05

P1 – certainty of difference with respect to the norm (before ischemia)

P2 – certainty of difference with respect to the control (without yeast RNA)

The data in Table 11 demonstrates that, during a short-term ischemia, the activity of NOS increased more than three-fold in the infarction zone (115 ± 40 and 186 ± 49 pmol/min on 1mg of protein accordingly, in the test and control groups). Hence, yeast RNA almost completely normalized the activity of NOS in the border zone of ischemic heart infarction, which may be one of the mechanisms of its cardio-protecting action.

Since cardiomyocytes contain both the inducible NOS isoform and its constituent isoforms (**Balligand J.L., Kobzik L., Han X., et al., Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endotelial (type III)**

Table 12.

Action of Yeast RNA on the Activity of NOS and Content of Arachidonic Acid in Rat Blood in Ischemia

	NOS Activity in picomol per 1 mg protein; M+-m; n=5			Content of free Arachidonic acid (nmol/1mg of protein)		
	Norm	Ischemia 30 min	Ischemia +Yeast RNA	Norm	Ischemia 30 min (control)	Ischemia +Yeast RNA
M +-m	30.35 3.40	14.22 1.43	26.45 3.73	0.77 0.13	0.24 0.04	0.48 0.02
P1		<0.01	<0.5		<0.01	>0.05
P2			<0.05			<0.01

P1 – certainty of difference with respect to the norm (before ischemia)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 12, the control group of animals demonstrated decreased levels of free AA more than three-fold (0,77 ± 1,43 and 30,35 ± 3,40 nmol/min on 1 mg of protein accordingly in normoxia and ischemia cases). The introduction of yeast RNA somewhat normalized the content of AA, by increasing it twice against the control value (P<0,001).

Table 13 indicates the content of free arachidonic acid in different heart zones in ischemia cases.

Table 13.

Action of Yeast RNA on the Content of Free Arachidonic Acid in Rat Heart in Ischemia (in nmol per 1 mg of protein; M-m; n=5)

	Ischemia 30 min (Control)				Ischemia 30 min+Yeast RNA		
	~~Intact (norm)~~ Norm	Border zone	Infarction zone	~~Together~~ Intact	Border zone	Infarction zone	~~Together~~ Intact
M +-m	4.827 0.378	9.910 1.003	9.716 0.947	9.813 0.919	7.270 0.456	8.530 0.741	7.900 0.493
P1		<0.01	<0.01	<0.001	<0.01	<0.01	<0.01
P2					>0.05	>0.05	>0.5

P1 – certainty of difference with respect to the norm (before ischemia)

Table 15.

Action of Yeast RNA Compound on the Activity of NOS in Rat Blood in the Dynamics of Adjuvant Arthritis (in picomol per 1 min per 1 mg of protein; M±m; n=5)

	Norm	Control			+Yeast RNA		
		I 3rd	II 8rd	III 14rd	I 3rd	II 8rd	III 14rd
M +-m	30.650 7.352	236.760 76.418	24.340 8.596	111.540 15.777	70.000 9.245	40.660 5.052	33.960 6.036
P1		<0.05	>0.5	<0.01	<0.02	<0.5	>0.5
P2					<0.02	<0.1	<0.01

P1 – certainty of difference with respect to the norm (in adjuvant arthritis)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 15, the control group of animals showed a substantial increase of NOS activity on the 3rd and 14th day of auto-immune pathology in comparison with norm (30,65 ± 7,35 picomol per 1 min per 1 mg of protein in norm, 236,76±76,42 picomol per 1 min per 1 mg of protein on the 3rd day, and 111,54±15,78 picomol per 1 min per 1 mg of protein on the 14th day). Such a significant increase in the activity of NOS indicates that activity of the inducible NOS-isoform (iNOS), whose synthesis is initiated by anti-inflammatory cytokines INF-γ, IL-1β, TNF-α, et. al., is the main compound in the calculated activity of NOS.

In the period between the 3rd (initiation of the auto-immune process) and 14th day (development of pathology), we observed a normalization in the activity of NOS in blood (24,34±8,60 pmol per 1 min per 1 mg of protein). This may probably be attributed to the activated protective reaction of body, and could be induced by inhibition of the expression of NOS as well as by modulation of the stability of its mRNA, or by inhibiting the process of its translation.

In the group of animals which took yeast RNA, initiation of the auto-immune process (on the 3rd day) was accompanied by a much smaller (in comparison with the control group) increase in the activity of NOS in blood (70,00±9,24 pmol per 1 min per 1

mg of protein against 236,76±76,42 pmol per 1 min per 1 mg of protein). Moreover, the activity of NOS decreased progressively over the next period in development of auto-immune process (40,66±5,05 pmol per 1 min per 1 mg of protein on the 8th day and 33,96±6,04 pmol per 1 min per 1 mg of protein on the 14th day).

Therefore, our tests on changes in the activity of NOS in rat blood in the course of an auto-immune process lead to the conclusion that yeast RNA is effective in decreasing the activity of iNOS in the course of an auto-immune process, both during its initiation and in the chronic stage. This property allows the use of yeast RNA in pathological conditions which are accompanied by iNOS induction: inflammatory processes, diabetes, atherosclerosis, tumour, hepatitis, infections, neuro-degenerate diseases (Parkinson's disease, Alzheimer's disease, multiple sclerosis, encephalitis), and others.

Example 6.3. Membrane-Protecting Action of Yeast RNA

The tests were conducted *in vivo* on the model of a chronic auto-immune process, which was accompanied by generation of a great quantity of free radicals (especially, nitric oxide) during the early stage of initiation. The membrane-protecting action of yeast RNA was studied by evaluating acid resistance of erythrocytes in the course of an auto-immune process. Acid resistance characterizes the wholeness of erythrocytal membranes. It increases in the chronic stage of different pathologies and decreases in the acute stage of development (process of initiation). For example, in the early period of development in inflammations, free-radical processes, which are induced by a generation of free radicals of oxygen and nitrogen, including nitric oxide generated by the inducible isoform of NOS (iNOS), are highly activated.

The level of damage in erythrocytes under the influence of various harmful factors in the course of an auto-immune process was evaluated by kinetic indicators of hemolysis, induced by a pH decrease in the environment. Kinetic indicators of hemolysis were recorded; the number of damaged cells was determined spectrophotometrically in

equal periods of time (30 s) by changes in the value of integral light dispersion of erythrocytal suspension (λ=750 nmol). Absorption spectra were registered by a spectrometer SF-26 (Russia). Acid lysis of erythrocytes was initiated by adding 10 µl of blood, which was diluted 20 times in the isotonic medium ~~0,14~~ 0.14 mol of NaCl + 0.01 mol of the citrate-phosphate buffer with pH=~~2,0 3,5~~ 2.0 3.5 (volume: 1 ml; density of erythrocytes in suspension: ~~0,7~~ 0.7x10^6 cells per ml). For such densities, the value of integral light dispersion of erythrocytes depends on the count, size, and shape of cells and is proportional to the number of cells in suspension.

Results are represented in the diagram of acid hemolysis of erythrocytes in Table 16 below, as the integral parameter of this process: total number of acid resistance of erythrocytes was calculated by summing up the products of the number of cells a_i which hemolyzed over the period of time a_j and t_j (total resistance (I) = $\Sigma a_i \cdot t_i$).

Decreased extinction levels on hemolysis diagrams represent the succession of erythrocytes with increased resistances entering hemolysis. Extinction starts decreasing usually 1.5 – 2 min later after a hemolytic injection (1 ml 0.004N HCl, which was prepared from 0.1N HCl and checked by titration). A lag-period of hemolysis is caused by a pre-hemolysis change in the form of erythrocytes (spherulation). Hemolysis of a single erythrocyte does not exceed 10 seconds. Hence, a 30-second interval between the measurements of existence levels excludes the possibility of counting twice the same erythrocyte undergoing lysis. It follows that, by the photometric registration of hemolysis kinetics, we can calculate, from the derived series of existences with intervals 30 seconds, the percentage of distribution of erythrocytes by resistance groups.

The change of existence from the beginning of hemolysis (E_b, t_b) to its final completion (E_e, t_e) is proportional to the number of all cells involved in hemolysis (100%), hence:

$$\Delta E = E_e - E_b = 100\%.$$